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Cardiac Fibrosis in Human Transplanted Hearts Is Mainly Driven by Cells of Intracardiac Origin

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Objectives The aim of our study was to determine the origin of collagen in the progression of myocardial fibrosis in human adult transplanted hearts.

Background Changes in the cardiac interstitial collagen network are thought to contribute to abnormal stiffness and function of the myocardium. The origin of fibrosis-mediating fibroblasts remains incompletely understood, and conflicting data from animal models suggest that they are either derived intracardially or immigrate from extracardiac sources.

Methods We studied endomyocardial biopsy specimens from 7 sex-mismatched (female donor heart to a male recipient) heart transplant recipients by a combination of chromogen in situ hybridization using probes specific for Y chromosomes with immunohistochemistry. On the basis of differences in genetic polymorphisms in the type III collagen gene between donor and recipient tissue, we quantitatively determined origin-specific type III collagen gene expression in fibrotic areas containing fibroblasts of putative extracardiac origin.

Results In areas of increased cardiac fibrosis years after heart transplantation, a substantial number of Y chromosome–positive spindle-shaped cells with a fibroblast-like appearance were detected. Many of these cells were identified as macrophages, and measurement of origin-specific type III collagen synthesis identified cells of intracardiac origin as the main source for collagen turnover in human cardiac fibrosis.

Conclusions Our data suggest that, in human myocardium, cardiac fibrosis due to chronic allograft rejection up to 15 years after transplantation or scar formation provoked by mechanical trauma is mainly driven by fibroblasts of intracardiac origin. Antifibrotic treatment strategies, therefore, should target molecular mechanisms that induce fibrillogenesis of cells with intracardiac origin. (J Am Coll Cardiol 2012;59:1008–16) © 2012 by the American College of Cardiology Foundation

Cardiac fibrosis is a common consequence of several pathological causes, including ischemia, inflammation, and episodes of cardiac rejection in transplanted hearts. The morphological changes of fibrosis are accompanied by increased stiffness of the heart and diminished contractility that lead to impairment of cardiac performance (1). On a molecular level, fibrosis is associated with a disruption in the context of the extracellular matrix and deposition of extracellular collagen. Fibroblasts are considered to be the major cellular mediators of these structural changes in the extracellular matrix architecture (2). However, the origin of these interstitial cells in the adult myocardium remains incompletely understood, and data are primarily derived from animal studies. Traditionally, it was assumed that fibroblasts in postnatal hearts were directly derived from embryonic mesenchymal cells and that the increase in the number of these cells is solely a result of proliferation of resident fibroblasts (3,4). However, this assumption has been challenged by the discovery that epithelial cells may transform into fibroblasts, a process called epithelial–mesenchymal transition (5). Moreover, in experimental animal models, it was demonstrated that extracardiac circulating progenitor cells can differentiate toward fibroblasts, thereby substantially contributing to intracardiac fibroblasts (6–8). Understanding the origin of fibroblasts in human myocardium and to what extent they contribute to cardiac fibrosis, may pave the way to future antifibrotic treatment strategies (9,10). Our study allowed us to detect the origin of collagen synthesis in human adult transplanted hearts. In contrast to previous studies (6–8) that either used animal models and/or genetically modified cells, we investigated transplanted human adult myocardium and used differences

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in single-nucleotide polymorphisms (SNPs) in the collagen type III gene to study the origin of collagen-producing cells (11). We quantified the origin of type III collagen under different pathological conditions: cardiac fibrosis caused by chronic allograft rejection up to 15 years after heart transplantation and areas of fibrosis caused by acute mechanical trauma. To our knowledge, we reveal for the first time the origin of collagen synthesis that is the major component of cardiac fibrosis in human adult hearts.

Methods

Patients and endomyocardial biopsies. Endomyocardial biopsy (EMB) results from 7 male patients who had received a female cardiac allograft during the years 1988 to 2008 were retrieved from the files of the Institute of Pathology, Medical University of Graz. The EMBs for monitoring rejection episodes had been performed either as routine biopsies or as biopsies taken when rejection episodes in the graft were suspected. The patients were maintained on a standard triple immunosuppression regimen (cyclosporine, azathioprine, and prednisolone), and rejection episodes assessed according to the International Society for Heart and Lung Transplantation were treated with pulse therapy of cortisone (500 mg daily for 3 days) or muromonabl-CD3 (OKT3), depending on the duration and severity of rejection and response to treatment. For each patient, we selected 4 biopsy specimens that showed histological signs of chronic allograft rejection in the transplanted heart. In addition, we explored origin-specific collagen type III synthesis in 6 biopsy specimens at 10 to 15 years after transplantation and selected 2 areas in each patient where an acute mechanical trauma (i.e., site of rebiopsy) had induced substantial cardiac scar formation.

Chromogenic in situ hybridization combined with CD68 immunohistochemistry. To identify whether recipient-derived fibroblasts populate areas showing signs of increased cardiac fibrosis, we selected samples based on the criteria mentioned earlier for Y chromosome–directed chromogenic in situ hybridization (CISH) analysis. Details of procedures and description of controls are provided in the Online Appendix. A conventional bright-field microscope was used to analyze the Y chromosome signals in different cell populations. Cell types were evaluated by using light microscopy for: 1) morphological features of the cell nuclei; 2) Y chromosome–positive staining as an indicator for recipient origin; and 3) CD68-positive cells. A cell was defined as Y chromosome positive if a punctate, dark-brown signal was localized within its blue-stained nucleus.

Immunohistochemical analysis of collagen type I and III and CD68. For assessment of the presence and the amount of collagen deposition as well as CD68-positive macrophages in EMBs, immunohistochemical detection of collagen type I and III and CD68 was performed in sequentially cut heart sections. (Details are described in the Online Appendix.)

Desoxyribonucleic acid isolation and genotyping of type III collagen. For determination of the genotypes of exon 30 (a single base substitution from guanine to adenine, rs1800255) and exon 32 (a single base substitution from cytosine to thymine, rs1801184) of the collagen type 3 alpha 1 (COL3A1) gene, we followed restriction fragment length polymorphism protocols as described in detail in the online Appendix. To determine the sensitivity and precision of the method, we performed dilution experiments by mixing deoxyribonucleic acid (DNA) from all genotype combinations with regard to the marker SNPs at 50:50%, 90:10%, 95:5%%, 99:1%, and 99.9:0.1% mixtures.

Abbreviations and Acronyms

CISH = chromogen in situ hybridization
COL3A1 = collagen type 3 alpha 1
DNA = desoxyribonucleic acid
EMB = endomyocardial biopsy
endoMT = endothelial–mesenchymal transition
mRNA = messenger ribonucleic acid
RNA = ribonucleic acid
SNP = single-nucleotide polymorphism
TGF = transforming growth factor

Ribonucleic acid isolation and origin-specific collagen type III expression. The marker SNPs of exon 30 and exon 32 of the COL3A1 gene from all 7 donor hearts and their corresponding recipient tissue were initially genotyped to determine differences in the donor and recipient collagen type III marker SNPs. In these patients, in whom the COL3A1 gene can be distinguished from recipients and the respective donor by the combination of at least 1 allele that is distinctive (thus enabling origin-specific detection of COL3A1 expression), we microdissected fibrotic areas of EMBs from each patient selected according to the criteria mentioned earlier. Details of procedures and control criteria for real-time polymerase chain reactions are detailed in the online Appendix. Reverse transcription was performed using the SuperScript II Reverse Transcriptase kit (Invitrogen GmbH, Lofer, Austria). For determination of the origin of the collagen type III messenger ribonucleic acid (mRNA), we used a primer extension and SNaPshot Multiplex Kit (Applied Biosystems, Vienna, Austria) as described in the online Appendix. Finally, labeled products were run on an ABI 3100xl capillary sequencer, and peak-heights of signals were measured using GeneMapper version 3.7 software (Applied Biosystems, Foster City, California).

Results

A detailed overview regarding the patients' characteristics and cardiovascular parameters 10 years after heart transplantation are shown in Tables 1 and 2. To determine whether extracardiac progenitor cells lead to fibroblast cell chimerism in transplanted human hearts, we performed CISH analysis to detect Y chromosomes in biopsies of microscopically confirmed areas of cardiac fibrosis and increased collagen

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Table 1 Patient and Biopsy Specimen Characteristics in Which Both CISH Analysis and Collagen Type III Origin Measurements Were Performed

Patient #	Reason for HTx	Age at HTx (yrs)	Interval to Biopsy (yrs)	Acute Rejection in Current Biopsy (ISHLT)	Documented Severe Rejection (ISHLT ≥3) Episodes at 10 Yrs Post-HTx	Post-Transplantation Graft Survival (yrs)
1	IHD	52	1	1A	0	Alive, 14 yrs post-transplantation
			4	1A		
			8	1A (site of rebiopsy)		
			9	1B		
			10	1A		
			12	0		
			13	0 (site of rebiopsy)		
			14	0		
2	DCM	52	1	1A	1	Alive, 11 yrs post-transplantation
			4	3B		
			8	0 (site of rebiopsy)		
			9	1A		
			10	1B		
			11	0 (site of rebiopsy)		
3	DCM	67	1	1B	0	Dead due to graft failure, 9 yrs post-transplantation
			3	1A		
			5	0 (site of rebiopsy)		
			6	1A (site of rebiopsy)		
			7	1A		
			9	0		
4	DCM	64	1	1A	1	Dead due to graft failure, 12 yrs post-transplantation
			4	3A		
			7	1B		
			10	0 (site of rebiopsy)		
			11	1A		
			12	0 (site of rebiopsy)		
5	DCM	57	1	1A	0	Alive, 14 yrs post-transplantation
			4	1B		
			9	0 (site of rebiopsy)		
			10	1B		
			11	1A		
			12	1A (site of rebiopsy)		
			13	1A		
			14	0		
6	DCM	43	1	1B	3	Dead due to graft failure, 19 yrs post-transplantation
			4	3B		
			10	0 (site of rebiopsy)		
			11	1A		
			13	0 (site of rebiopsy)		
			14	1A		
			14	0		
			15	0		
7	DCM	17	Not analyzed	No differences in collagen genotype		Alive, 13 yrs post-transplantation

CISH = chromogen in situ hybridization; DCM = dilated cardiomyopathy; HTx = heart transplantation; IHD = ischemic heart disease; ISHLT = International Society for Heart and Lung Transplantation.

type I and III deposition (Fig. 1) selected according to the criteria described in the Methods section. Among the sex-mismatched transplant patients, Y chromosomes were detected in some nuclei of cardiomyocytes and endothelial cells in all samples. However, chimerism was rare, and only <0.5% of all cardiomyocytes were Y chromosome–positive cells. In areas of increased collagen deposition (Figs. 2A and 2D), we analyzed sequentially cut heart sections and stained for CD68-positive macrophages (Figs. 2B and 2E) followed by the Y chromosome–directed CISH technique (Figs. 2C and 2F). In fibrotic areas (Fig. 2A), where only a sparse infiltrate of macrophages was visible (Fig. 2B), nearly all ovoid- to spindle-shaped putative fibroblasts were Y chromosome negative (Fig. 2C) and thus of donor (intracardiac) origin. In contrast, areas of fibrosis (Fig. 2D) containing a high number of CD68-positive macrophages (Fig. 2E) showed cells with ovoid Y chromosome–positive nuclei (Fig. 2F). Indeed, combined CISH and CD68 staining

Table 2 Cardiovascular Parameters 10 (±1) Years Post-Transplantation

Patient #	NYHA Class (1–4)	TR (0–3)	LVEF (%)	LVEDD (mm)	DD (1-4)	IVS (mm)	Hypertension (>140/90 mm Hg)
1	2	1	56	52	1	16	Yes
2	1	1	67	46	1	9	No
3	2	2	49	42	2	15	Yes
4	2	2	69	42	2	14	Yes
5	1	1	65	43	1	12	Yes
6	2	2	69	49	2	17	Yes
7	1	0	61	50	1	12	No

DD = diastolic dysfunction determined according to transmitral blood flow; IVS = end-diastolic diameter of intraventricular septum; LVEF = left ventricular ejection fraction; LVEDD = left ventricular end-diastolic diameter; NYHA = New York Heart Association; TR = tricuspid regurgitation (0 = none, 1 = mild, 2 = moderate, and 3 = severe).

identified many of these cells as Y chromosome and CD68-positive macrophages (Fig. 3A), whereas ovoid- to spindle-shaped, CD68, and Y chromosome–negative cells represent donor-derived fibroblasts (Fig. 3B). Table 3 provides an overview of the number and percentages of positive cells in each patient.

For determination of whether and to what extent fibroblasts of putative extracardiac origin contribute to cardiac fibrosis, we developed a strategy to detect origin-specific fibrosis (Fig. 4). In our white sample collection, the frequency of the G allele was 0.71, and the frequency of the A allele was 0.29 for exon 30; the frequency of the T allele was 0.82, and the frequency of the C allele was 0.18 for exon 32. In 6 of the 7 patients, the genotypes between donor and recipient tissue showed at least 1 distinctive allele (Table 4). For further COL3A1 expression analysis, we included only these 6 patients for whom at least 1 allele differed between donor and recipient tissue. For technical reasons, it was necessary to establish a correction factor derived from genomic DNA, in which the ratio of alleles is known to be equimolar (11). For each individual genotype combination, we measured the genomic ratios of the A and G alleles and the T and C alleles. Dilution experiments showed that, depending on the genotype, 0.5% to 5% of recipient-derived nucleic acids are reproducibly quantifiable by using this technique in the background of the dominating allele (Table 4). Overall, 48 biopsy specimens were analyzed for origin-specific COL3A1 mRNA expression (Online Table 1). None of the 24 biopsy specimens showing increased fibrosis from chronic rejection, the 6 specimens harvested 10 to 15 years after heart transplantation, the 6 specimens harvested between day 3 and day 30 after transplantation, or the 12 specimens showing scar formation after acute mechanical trauma (i.e., site of rebiopsy) indicated any detectable level of COL3A1 mRNA from extracardiac origin (Fig. 5), with a theoretically 0.1% detection limit for the assay. (For details, see the Online Appendix.)

In an attempt to investigate whether macrophages influence the number of extracardiac fibroblasts in areas of cardiac fibrosis or induce collagen synthesis of cells with recipient origin, we analyzed areas of fibrosis that showed high numbers of CD68-positive macrophages (Fig. 2E) and compared them with areas of fibrosis containing only a few numbers of macrophages (Fig. 2B). We found no differences in the number of recipient-derived fibroblasts or in the amount of recipient-derived collagen synthesis.

Discussion

Generally, the pool of cardiac fibroblasts may be recruited from different sources. Traditionally, there is a well-defined sessile intracardiac fibroblastic cell population consisting of fibroblasts that only proliferate after receiving an activating stimulus. Undifferentiated intracardiac fibroblastic progen-

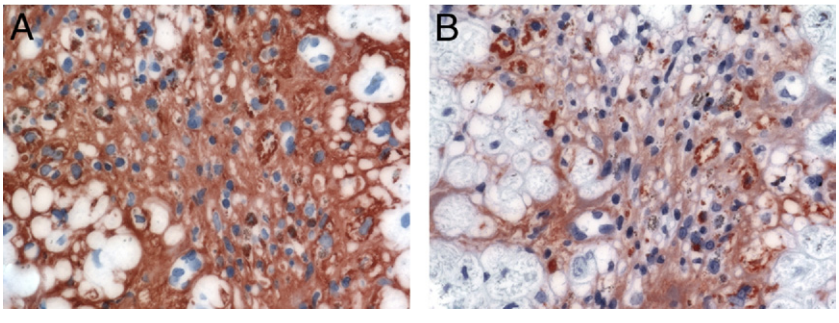

Figure 1 Immunohistochemical Analysis of an Area of Increased Fibrosis

(A) Collagen type I deposition and **(B)** collagen type III deposition. Specific antibodies decorating collagen fibers are visualized by a **red** chromogen; cell nuclei are counterstained by **blue** hematoxylin.

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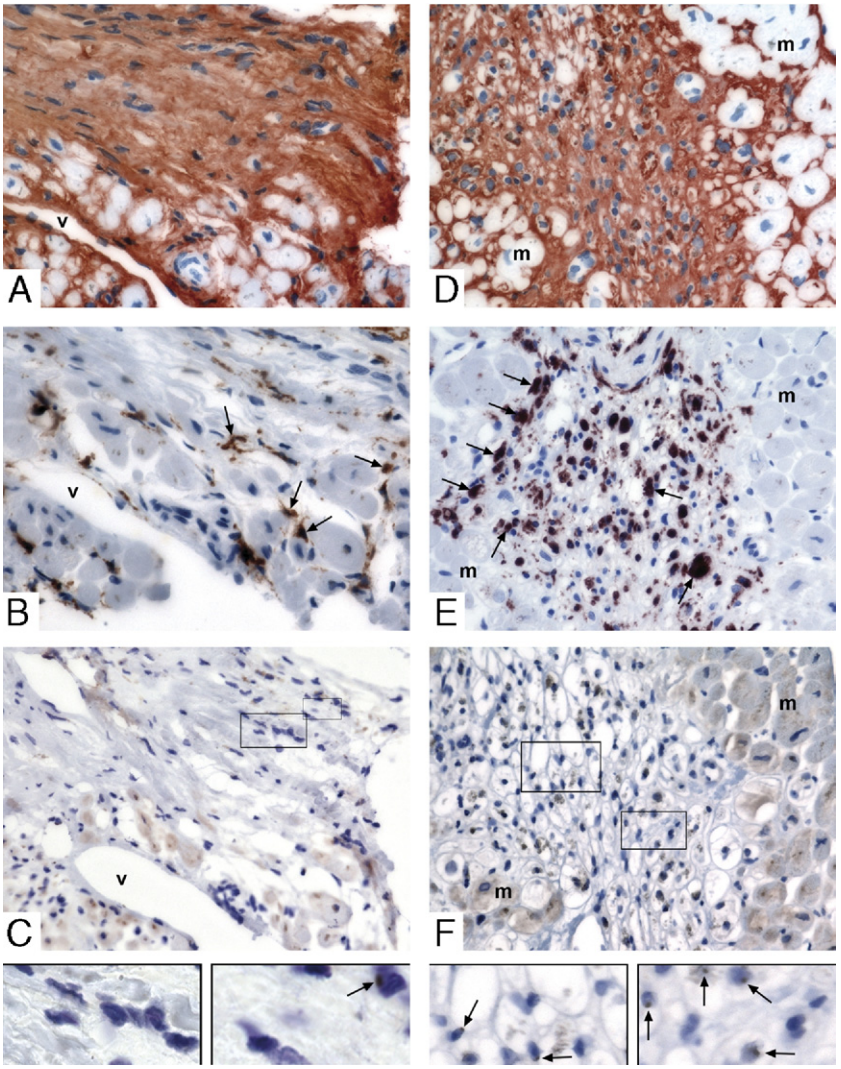

Figure 2 **Sequentially Cut Heart Sections Showing Areas of Increased Fibrosis**

Immunohistochemical **(red)** staining for collagen **(A, D)**, CD68 staining with a **red** chromogen **(B, E)** and Y-chromosome in situ hybridization **(C, F)**. **Insets below C and F** show higher magnifications of Y chromosome–positive nuclei, indicated by an **arrow** as a punctate brown signal within the nucleus. **A to C** contain a longitudinally cut blood vessel (v), and **D to F** show surrounding cardiomyocytes (m). In areas of **(A)** increased fibrosis that showed sparse staining of CD68-positive macrophages (**arrows in B** indicate CD68-positive cells), most ovoid- to spindle-shaped cells are Y chromosome negative; thus, they are donor derived and most likely represent fibroblasts. An **arrow in the right inset of C** indicates a single Y chromosome–positive cell. In contrast, in areas of **(D)** increased fibrosis showing abundant infiltrations of CD68-positive macrophages (**arrows in E** indicate CD68-positive cells), many of the ovoid- to spindle-shaped cells show Y chromosome–positive nuclei **(arrows in insets below F)**.

itor cells are present in normal newborn and adult hearts, but their differentiation fate and role in cardiac fibrosis are poorly understood (12). It has been shown that, similar to the embryonic developmental concept, a mechanism in which endothelial cells may emigrate and transform into intracardiac fibroblasts might represent an important source of intracardiac fibroblasts (5). Another potential source of fibrosis-mediating fibroblasts might be extracardiac progenitor cells that emigrate from the circulation to different organs and substantially contribute to organ fibrosis under pathological conditions (13,14). In this study, we examined whether fibroblasts of extracardiac origin are enriched in fibrotic areas caused by different pathological conditions in human cardiac allografts. For this purpose, we first followed a strategy to detect fibroblasts with putative extracardiac origin in human transplanted hearts by studying cell chimerism in a model of sex-mismatched cardiac transplantation. Based on this model, Minami et al. (15) demonstrated a high degree of chimerism for different nonmyocytic cardiac cell types (e.g., endothelial cells with up to 24% originating from extracardiac sources 1 month after transplantation). One major obstacle for studying fibroblast cell chimerism, as stated by Minami et al. (15), is that no specific antibody for formalin fixed paraffin embedded tissue exists that can

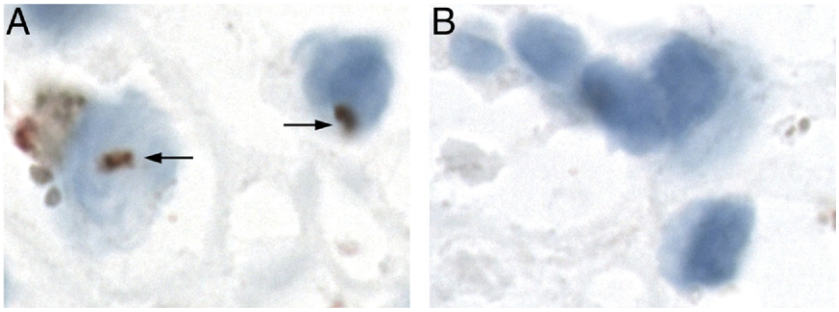

Figure 3 Combined Y Chromosome CISH Analysis With Immunohistochemical Staining for CD68

In an area of increased fibrosis, ovoid- to spindle-shaped nucleus shows a punctate brown signal in the nucleus **(arrow)** and red staining for CD68, thus indicating a male recipient–derived macrophage. **(A)** Multiple ovoid- to spindle-shaped, CD68-negative, and Y chromosome–negative nuclei are visible in this area of cardiac fibrosis **(B)**. CISH = chromogen in situ hybridization.

reliably detect fibroblasts. In our study, we chose to use CISH technology, which allows for simultaneous observation of tissue morphology and Y chromosome signals. By immunohistochemically preselecting areas with prominent collagen type I and III protein deposition, we encountered a substantial number of cells with a spindle-shaped fibroblast-like phenotype that displayed a positive signal for the Y chromosome. However, combining CISH analysis with immunohistochemical staining for the CD68 antigen demonstrated that many of these fibroblasts with putative extracardiac origin were in fact inflammatory macrophages. In areas of fibrosis, where we excluded the presence of macrophages by a negative selection of CD68 staining, the vast majority of cells of putative fibroblastic phenotype were Y chromosome–negative cells. Activated macrophages have been previously implicated to cause incorrect chimerism results because they may express some markers typical for cardiomyocytes or fibroblasts (16). In this context, it should be mentioned that under some conditions, monocyte-derived, collagen-synthesizing fibrocytes might also express CD68 (17). Previously published studies have reported controversial results regarding the proportion of fibroblasts of extracardiac origin in cardiac fibrosis under different pathological conditions. For instance, Wu et al. (7) reported that up to 65% of fibroblasts were of recipient origin in a rat model of chronic allograft rejection and that circulating recipient-derived mesenchymal stem cells may substantially repopulate intracardiac fibroblasts (6). In mice at 3 to 12 months after bone marrow transplantation, Visconti and Markwald (8) demonstrated that hematopoietic stem cell–derived cells contribute to fibroblast formation under normal (nonpathological) conditions. The same researchers also found that periostin plays an essential role in directing the differentiation of progenitor cells to mature into fibroblasts (12). Furthermore, Möllmann et al. (18) reported a bone marrow–derived fibroblast content of 24% at 7 days and 28% at 21 days after myocardial infarction in the myocardium of a transgenic mouse model. In a murine model of nonadaptive fibrosis, Haudek et al. (19) reported that ischemic cardiomyopathy is associated with monocyte chemoattractant protein-1 driven uptake of bone marrow–derived blood-borne fibroblast precursors of hematopoietic origin.

Table 3 Counting of Y Chromosome–Positive/Negative Spindle- to Ovoid-Shaped Nuclei in Areas of Increased Fibrosis

	Y Chromosome CISH Staining	Combined Y Chromosome/ CD68 Staining
Patient #	No. (%) of Y Chromosome–Positive/ Y Chromosome–Negative Spindle- to Ovoid-Shaped Nuclei	No. of Y Chromosome–Positive CD68-Positive Cells/ Y Chromosome–Positive CD68 Negative Cells/ Y Chromosome–Negative CD68-Negative Cells
1	41/610 (6.7)	31/2/510
2	52/480 (10.8)	41/0/460
3	61/750 (8.1)	37/3/520
4	15/630 (2.3)	28/2/410
5	31/680 (4.5)	19/1/490
6	83/560 (14.8)	33/3/550
7	68/800 (8.5)	49/4/550

CISH = chromogen in situ hybridization.

In contrast to these studies that show a substantial proportion of fibroblasts with extracardiac origin, Zeisberg et al. (5), who used a genetically engineered mouse model, demonstrated that only 13.4% of FSP1-positive (a marker for fibroblastic differentiation) cells originated from circulating bone marrow–derived cells in hearts subjected to pressure overload by aortic constriction. In unbanded control hearts, the authors did not detect any FSP1-positive cells of bone marrow origin. In another murine model for myocardial infarction, Yano et al. (20) found only a few vimentin-positive putative fibroblasts of bone marrow origin at 7 days post-coronary artery occlusion. The reasons for these discrepancies are not known; however, they may be due to differences in fibrosis-mediating triggers, use of genetically modified cells, the timing of the observations,

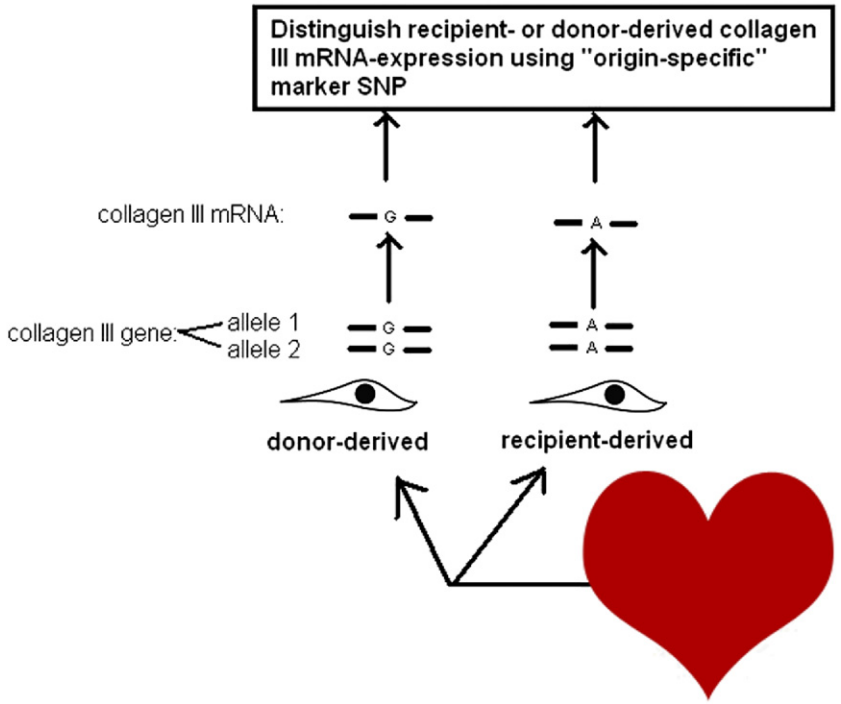

Figure 4 Strategy for Determining the Origin-Specific Type III Collagen Expression in Transplanted Hearts

Differences of single-nucleotide polymorphisms (SNPs) in the coding region of the collagen III gene enable the measurement of recipient and donor-derived collagen III messenger ribonucleic acid (mRNA) expression.

interspecies differences, and the use of different markers and methods for identifying fibroblasts. In addition, the use of immunosuppressive agents in human patients could contribute to the observed discrepancy. Whether and which of these experimental animal models sufficiently reflect the human situation remains unclear. Thus, the aim of our study was not only to identify the phenotype of fibroblasts with putative extracardiac origin but also to determine whether and to what extent these fibroblasts have an active functional role in the development of cardiac fibrosis in human hearts.

Table 4 Allele Constitution at 2 Loci of the COL3A1 Gene in Donor and Recipient Tissues

Sample Identification	COL3A1 rs1800255 (Exon 30 G>A)	COL3A1 rs1801184 (Exon 32 T>C)
Recipient 1	G/G	T/T (5%)
Donor 1	G/G	C/T
Recipient 2	G/A (0.5%)	T/T
Donor 2	A/A	T/T
Recipient 3	G/A (2.5%)	T/T
Donor 3	G/G	T/T
Recipient 4	G/A (2.5%)	T/T (5%)
Donor 4	G/G	C/T
Recipient 5	G/A (2.5%)	T/T (2.5%)
Donor 5	G/G	C/C
Recipient 6	G/A	C/T (2.5%)
Donor 6	G/A	T/T
Recipient 7	G/G	T/T
Donor 7	G/G	T/T

All recipients except Patient #7 can be distinguished from the respective donor by the combination of at least 1 allele that is distinctive, thus, enabling origin-specific detection of collage type 3 alpha 1 (COL3A1) expression. The limits of detection for each possible allele combination obtained from serial dilution experiments are depicted in parentheses.

Collagen type III represents one of the major constituents of cardiac fibrosis, and cardiac fibroblasts are the predominant collagen producers in the heart (21). The serum concentration of the propeptide of type III procollagen has been used as a marker for allograft rejection because collagen type III synthesis reflects wound healing and collagen turnover after rejection episodes (22). Therefore, we measured origin-specific collagen type III synthesis under different pathological conditions. For the purpose of detecting and quantifying origin-specific collagen synthesis, we adapted a technique that has been used previously to quantify allelic expression imbalances (23). The use of 2 marker SNPs that are part of the coding sequence of COL3A1 mRNA enabled us to quantify origin-specific type III collagen. Based on differences in the marker SNPs between donor and recipient collagen, we measured the origin-specific COL3A1 mRNA expression in myocardial tissue with increased cardiac fibrosis. Interestingly, in all samples that we tested, we could only detect COL3A1 mRNA of donor origin. These data suggest that, in contrast

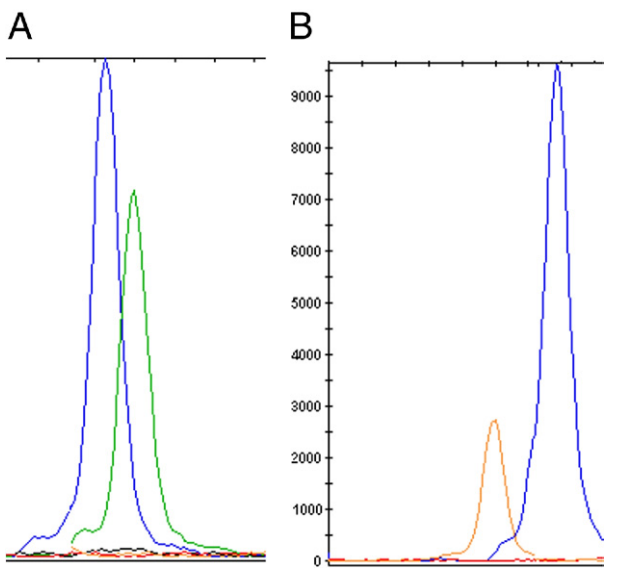

Figure 5 **Determination of Origin-Specific COL3A1 mRNA Using the SNaPshot Assay**

(A) A positive control from a heterozygous sample in which both alleles (G allele in **blue** and A allele in **green** from exon 30) are present in equimolar amounts. For technical reasons, the alleles show peaks with slightly different heights (29). Due to the influence of the dye on the mobility of the desoxyribonucleic acid fragment, the alleles show no exact overlap. **(B)** Representative data from analysis of collagen type III expression in donor heart of patient 3. Only **peaks** in **blue color**, representing G allele from donor-derived collagen, were detected in all biopsy specimens analyzed (**yellow peak** represents an internal size marker of the GeneScan 120 LIZ size standard [ABI]). COL3A1 = collagen type 3 alpha 1; mRNA = messenger ribonucleic acid.

to previous reports (6,7) from animal models, fibroblasts of intracardiac origin that are either pre-existing or generated by endothelial–mesenchymal transition (endoMT) are the main source of collagen in cardiac fibrosis in human cardiac allografts. We observed no evidence of type III collagen synthesis from extracardiac origin, up to 15 years after cardiac transplantation or in areas with increased content of inflammatory cells. It therefore seems implausible that circulating cells substantially contribute to remodeling or normal collagen homeostasis in the human adult heart.

Study limitations. A potential limitation of our study is the relative small sample size, especially with regard to biopsies conducted early after transplantation. Early onset of fibrosis by fibroblasts of extracardiac origin was not supported by our study. However, as the majority of our biopsy specimens were obtained relatively late after transplantation, the small sample size in the "early" subset of biopsies does not allow us to completely rule out this possibility.

Currently, there is no therapy available that reverses cardiac fibrosis once significant amounts of collagen have been deposited. Thus, there is paramount interest to target its source: the cardiac fibroblast. Our study identified that the majority of cardiac collagen is synthesized by fibroblasts of intracardiac origin. Previous studies reported that a substantial part of intracardiac fibroblasts—especially in diseased conditions such as pressure overload hypertrophy, failure, or graft versus host reaction—can be derived by endoMT (5,24). Thus, interfering with endoMT may open new therapeutic avenues to prevent cardiac fibrillogenesis. Transforming growth factor (TGF)-beta signaling, both with its canonical (SMADs) and noncanonical (e.g., MEK, p38-MAPK, PI3K, TAK1) pathways, plays an important role in the process of endoMT, the fibroblast-to-myofibroblast transition, and in adverse fibrotic remodeling in general (25–30). For instance, inhibition of SMAD3, either by small molecule inhibitors or transgenic mouse models, modulates endoMT; BMP7 prevents endoMT and is itself modulated by TAK1 (24,26,27,31). Although these interventions are still only used in basic research, TGF-beta inhibition through anti–TGF-beta antibodies is already under investigation in clinical trials for a variety of diseases. Interestingly, there is also a cross-talk between atrial natriuretic peptide/cyclic guanosine monophosphate/protein kinase G and TGF-beta signaling, in which protein kinase G phosphorylates SMAD3 on 2 sites that are distinct from the TGF-beta receptor kinase phosphorylation sites, thereby inhibiting nuclear translocation of pSMAD3 (32). This is intriguing, as PDE5a inhibition is currently evaluated for diastolic dysfunction and is an established therapy for pulmonary hypertension, both conditions that are frequently encountered in patients after heart transplantation (33,34).

Conclusions

Although we cannot exclude the possibility that fibroblast cell chimerism might occur to a limited extent in the transplanted human myocardium, the functional contribution of cells with extracardiac origin to cardiac fibrosis is minimal or absent in chronic allograft rejection up to 15 years after transplantation. According to our data, cardiac chimerism of fibroblasts is not associated with chronic graft failure. These data refute the hypotheses derived from some animal studies. Therefore, therapeutic approaches for inhibiting cardiac allograft fibrosis and remodeling of the heart should target mechanisms that stimulate intracardiac fibrillogenesis rather than mechanisms which restrict the immigration of circulating fibroblastic progenitor cells to the myocardium.

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Key Words: cardiac fibrosis ■ chimerism ■ fibroblasts.

APPENDIX

For supplementary materials, please see the online version of this article.